of lifetime changes with spatial localization. Here we describe an application of FRET image analysis using the Rac and RhoA biosensors in which the specific distribution of the sensor in a cell is important to establish its activation. Specifically we study cells in a 3D matrix in which the activation of the Rac and RhoA biosensor could have a different distribution than in 2D. In order to simultaneously measure several 3D locations we use a method in which we measure FRET along a 3-dimensional line which encompasses different parts of the cell. The measurement of the decay at each point of a 3D line can be done very fast (in millisecond) potentially revealing the dynamics of the biosensor at a time scale that is of particular significance for cellular reactions. When the laser spot is moving along the line, we can also do measurements at two emission wavelengths giving us the chance to compare the phasor-FRET determination with the ratiometric method.
The authors acknowledge financial support from the National Institutes of Health P50 GM076516 and P41 RR03155

1184-Plat
Polarized Fluorescence Correlation Spectroscopy (pFCS): A Single-Molecule Method for Simultaneously Measuring Homo-FRET, Brightness, and the Diffusion of Protein Complexes in Living Cells
Tuan A. Nguyen, Pabak Sarkar, Jithesh V. Veetil, Srinagesh V. Koushik, Christopher Thaler, **Steven S. Vogel**.
NIAAA/NIH, Rockville, MD, USA.
FRET is a phenomenon where excited state energy of a fluorescent donor molecule is transferred, by a non-radiative dipole-dipole coupling mechanism, to a nearby acceptor. Because the efficiency of FRET provides information regarding the distance separating the donor and acceptor, FRET is used to study protein interactions in living cells. In conjunction with automated microscopy, FRET can also be used to screen for drugs that perturb specific protein-protein interactions. The utility of FRET, however, is limited by the possibility of both false positive (caused by over expression and molecular crowding), and by false negative signals (resulting from separation distances greater than 10 nm and from very low dipole orientation factors). To overcome these limitations we have developed Polarized Fluorescence Correlation Spectroscopy (pFCS), a single-molecule based method to characterize the interactions of proteins in complexes. Using pFCS, Homo-FRET (a 1-10 nm proximity gauge), brightness (a measure of the number of fluorescent subunits in a complex), and the lateral diffusion coefficient (an attribute sensitive to viscosity, mass, and the shape of a protein complex) can be simultaneously measured. With these measurements, the interpretation of FRET can be rigorously constrained thus reducing the likelihood of both false negative and false positive interpretations. Standards consisting of tandem covalently linked concatemers of between 1 and 6 Venus molecules were used to validate pFCS in both solution and in cells. The utility of pFCS was demonstrated by measuring differences in Homo-FRET, subunit stoichiometry and correlation time between Venus-tagged CaM-kinase-II holoenzyme (α or β).

1185-Plat
Dynamics of DNA Mismatch Repair Revealed by Single Molecule FRET
Keith Weninger.
North Carolina State University, Raleigh, NC, USA.
Single molecule FRET (smFRET) has rapidly gained popularity because it can provide unique information about biomolecular systems. In particular, smFRET provides quantitative nanoscale resolution of dynamic molecular motions and multimolecular interactions, all for unsynchronizable samples that may have multiple reaction pathways operating in parallel. As the experimental capabilities of smFRET advance, this method has been applied to increasingly complex biological systems. I will illustrate these complex system advantages of single molecule FRET by presenting measurements of DNA mismatch repair proteins MutS and MutL interacting with mismatched DNA. These proteins are the initial sentries that detect single base mismatches and insertions/deletions and activate repair cascades. We use smFRET to determine dynamic DNA bending by MutS, concomitant conformational changes within MutS itself, motion of MutS scanning along DNA, ATP binding states that commit MutS:mismatch DNA complexes to convert to sliding states used in signaling, and the modulation of these MutS behaviors by interactions with MutL.

1186-Plat
Single-Molecule FRET: Theory and Analysis of Photon Sequences
Irina V. Gopich, Attila Szabo.
National Institutes of Health, Bethesda, MD, USA.
Single-molecule FRET measurements contain information on conformational dynamics because the rate of energy transfer depends on the distance between donor and acceptor labels attached to a molecule. The output of such measurements is a sequence of photons of different colors separated by apparently random time intervals. In addition, the delay time between laser pulse and photon arrival can be recorded. To extract information from such raw data, it is necessary to understand in detail all the complex microscopic processes involved. We consider various quantitative methods to analyze sequences of photons emitted by a molecule with interchanging conformational states. Photon sequences with recorded interphoton times can be analyzed by maximizing the appropriate likelihood functions with respect to the parameters of a model of the conformational dynamics. The consistency of the model with the data can be checked by recoloring the photons trajectory and comparing the predicted and observed FRET efficiency histograms. These photon-by-photon methods are rigorous for both immobilized and diffusing molecules. Binned photon sequences, in which only the numbers of donor and acceptor photons in consecutive time intervals are recorded, can be analyzed by constructing FRET efficiency histograms or, alternatively, by analyzing the whole sequence of photon counts using likelihood-based methods. For the FRET efficiency histograms, we derive accurate multi-Gaussian approximations without any adjustable parameters when the molecule has multiple conformational states. For the whole sequence analysis, we provide approximate likelihood functions for the binned photon sequences. It is shown how these methods can be extended to include information from photon delay times.

1187-Plat
Structural Modeling of Full Length Maguk Scaffold Proteins using Single Molecule FRET Restraints
Mark E. Bowen.
Stony Brook University, Stony Brook, NY, USA.
By conjoining protein interaction domains, scaffold proteins form a framework to organize signaling. Synaptic MAGUK scaffold proteins contain three PDZ domains, an SH3 domain and an inactive Guanylate kinase (GK) domain, which are connected by flexible linkers. We used single molecule fluorescence to probe the structure of MAGUK proteins in their isolated ''ground'' state. We found that the five domains partition into two independent units with relatively fixed structures. From these findings, it is unclear how allosteric coupling between the PDZ domains would be possible. Comparative structural analysis showed a conservation of the PDZ domain organization but the differences within the PDZ3-SH3-GK module. Surprisingly, we found no signs of conformational heterogeneity, transitions or dynamics on the microsecond to second timescale. These findings provide the first unambiguous assignment of domain positioning in a full length scaffold protein.

1188-Plat
From Force-Fields to Photons: MD Simulations of Dye-Labeled Nucleic Acids and Monte Carlo Modeling of FRET
Peker Milas, Benjamin D. Gamari, Louis Parrot, **Lori S. Goldner**.
University of Massachusetts, Amherst, MA, USA.
Fluorescence resonance energy transfer (FRET) is a powerful technique for understanding the structural fluctuations and transformations of RNA, DNA and proteins. Molecular dynamics (MD) simulations provide a window into the nature of these fluctuations on a different, faster, time scale. We use Monte Carlo methods to model and compare FRET data from dye-labeled RNA with what might be predicted from the MD simulation. With a few notable exceptions, the contribution of fluorophore and linker dynamics to these FRET measurements has not been investigated. We include the dynamics of the ground state dyes and linkers in our study of a 16mer double-stranded RNA. Water is included explicitly in the simulation. Cyanine dyes are attached at either the 3' or 5' ends with a 3 carbon linker, and differences in labeling schemes are discussed.

1189-Plat
High Precision FRET to Determine Dynamic Protein Structures
Claus A. Seidel[1], Stanislav Kalinin[1], Simon Sindbert[1], Thomas Peulen[1], Yathrib Ajaj[1], Paul J. Rothwell[1], Carola Hengstenberg[2], Christian Herrmann[2].
[1]Heinrich-Heine University Duesseldorf, Duesseldorf, Germany,
[2]Ruhr-University Bochum, Bochum, Germany.
Measuring in solution and utilizing the single-molecule advantage of fluorescence detection we established a toolbox to generate FRET-constrained structure models of biomolecules which can also show their heterogeneity and flexibility. Our approach comprises seven steps: **(1)** Quantitative measurement of FRET by multiparameter fluorescence detection of single molecules [1]; (2) Rigorous analysis and error determination of FRET derived donor-acceptor distances by analyzing the photon distributions and time resolved anisotropies of

the dyes; (3) Appropriate description for the spatial distribution of the fluorophore by fast accessible volume (AV) simulations [2] to determine the dye positions relative to the biomolecule; (4) Search for possible structures via a FRET positioning system using a spring-network algorithm. Possible structures are generated either by a model-based approach with rigid body docking or model free by selecting suitable models from a huge structure library; (5) Docking is repeated many times to find all possible arrangements and assure the completeness of generated structural ensemble; (6) The obtained models are ranked according to their violation of FRET constraints and steric clashes. Then they are assigned to clusters of related structural organization in order to judge the uniqueness of structural models; (7) The precision (RMSD) of the structure models is determined using a bootstrapping procedure. We demonstrate the accuracy of high-precision (hp) FRET in two experiments - determination of the DNA position in HIV-1 reverse transcriptase:primer/template complexes and arrangement of a primer/template DNA bound by HIV-1 reverse transcriptase and analysis of the internal structural heterogeneity of human guanylate binding protein 1 (hGBP1). These studies show that hpFRET studies are valuable tool to complement the structure information obtained by classical methods.

[1] Sisamakis, E., et al.; Methods in Enzymology **475,** 455-514 (2010).
[2] Sindbert, S., et al.; J. Am. Chem. Soc. **133**, 2463-2480 (2011).

Platform: Interfacial Protein-Lipid Interactions I

1190-Plat
Visualization of Supported Lipid Bilayer Remodelling by s-mgm1 using Correlated Confocal Fluorescence and Atomic Force Microscopy
Michael K. Wong, Jarungjit Rujiviphat, G. Angus McQuibban, **Christopher M. Yip**.
University of Toronto, Toronto, ON, Canada.

In yeast, the GTPase s-mgm1 is responsible for the fusion of inner mitochondrial membranes, a process essential for maintenance of normal mitochondrial morphology and function. Direct, real-time visualization of the effects of s-mgm1 upon mitochondrial mimic membranes is particularly relevant to elucidating the mechanism by which it acts. Here, we utilize both confocal microscopy and AFM to demonstrate that s-mgm1 spontaneously induces GTP-independent pinching and tubulation of lipids in the gel phase. Subsequent addition of GTP causes further remodelling of the membrane. Similar experiments using ATR-FTIR suggest that the membrane induces increased order in protein conformation. Our data is consistent with a model by which s-mgm1 promotes fusion of opposing membranes by pinching and tubulation.

1191-Plat
Controlled Protein Confinement in Phase-Separated Membranes Tethered onto Micro-Patterned Supports
Friedrich Roder, Sharon Waichman, Oliver Beutel, Dirk Paterok, Jacob Piehler.
University of Osnabrueck, Osnabrueck, Germany.

Phase-separation of lipid membranes into liquid-disordered (l_d) and liquid-ordered (l_o) domains has been recognized as a key principle for the functional organization of the plasma membrane. In classic model systems such as GUVs, the spatial organization of phase separated membranes is a stochastic, time-dependent process, which depends on the lipid composition and often leads to a complete coalescence of the lipid phases. We have here established an approach for a spatial control of lipid phase separation in tethered polymer-supported membranes (PSM). On a dense poly(ethylene glycol) polymer brush functionalized with hydrophobic tethers, contiguous, highly fluid PSM were obtained by means of fusion of SUVs.[1] Free diffusion of lipids and reconstituted transmembrane proteins in these PSM was confirmed by FRAP, FCS and single molecule tracking. Strikingly, phase separation of ternary lipid mixtures (DOPC/SM/cholesterol) in PSM into l_d and l_o phases was dependent on the properties of the anchoring group. We exploited these features for assembly of l_o domains into defined structures using micropatterned tethers. Within isolated micropatterns, l_d and l_o phases self-assembled into stable, reproducible membrane architectures. By binary micro-patterning of different tethering groups into complementary areas, ternary lipids mixtures separated into l_o and l_d phases controlled by the geometry of the underlying tethers. Transmembrane proteins reconstituted in these phase-separated PSM strictly partitioned into the l_d phase. Hence, the l_o phase could be used for confining transmembrane proteins into microscopic and submicroscopic domains. The permeability of these barriers for lipids and proteins and thus their exchange between adjacent l_d compartments can be globally and locally controlled by the temperature. These features have been exploited for probing interactions and diffusion of a transmembrane receptor in the context of l_d and l_o phases.

1) Roder, F.; et al. Anal Chem 2011, 83, 6792-6799.

1192-Plat
Lipid-Protein Interactions in Nanodiscs: *How to Enhance Stability*
Maria H. Wadsäter[1], Marité Cardenás[1], Stine Rønholt[2], Selma Maric[2], Nicholas Skar-Gislinge[2], Søren R. Midtgaard[2], Lise Arleth[2], Kell Mortensen[2], Robert O. Ryan[3], **Jens B. Simonsen**[2,3].
[1]Nano-Science Center, University of Copenhagen, Copenhagen, Denmark, [2]Nanobioscience, University of Copenhagen, Frederiksberg, Denmark, [3]CHORI, Oakland, CA, USA.

Lipid-protein interactions can function as ''co-factors'' that affect the properties / function of transmembrane proteins. Herein, the interaction between anionic dimyristoylphosphatidylglycerol (DMPG) and zwitterionic dimyristoylphosphatidylcholine (DMPC) with the amphiphatic membrane scaffold protein (MSP), were studied. Two 25 kDa MSP wrap around the circumference of discoidal bilayer in a belt-like manner to form a nanodisc [1,2]. The membrane-like structure of nanodiscs has been used for reconstitution of membrane proteins in a native-like environment. Differential scanning calorimetry was employed to characterize lipid-protein interactions in these particles by evaluating changes in MSP denaturation temperature and lipid gel-liquid phase transition as a function of nanodisc lipid composition and ionic strength. Small-angle X-ray scattering and size-exclusion chromatography were used to determine the overall structure of the nanodisc. We suggest the nanodisc lipid is divided into a lipid rim that interacts with the internal face of the MSP helical segments, while the centrally located nanodisc lipids maintain a more bulk-like lipid behavior. This finding is important for reconstitution of membrane proteins since the presence of a 'lipid rim' serves to prevent contact between the membrane protein and the MSP. Furthermore, the presence of two distinct lipid environments reduces the available area for reconstituted membrane proteins in the nanodisc. We also show that the negatively charged DMPG has a higher preference for the rim due to its negatively charged headgroup. Finally, we conclude that DMPG stabilizes the nanodisc in a twofold manner: i) DMPG 'freezes' the MSP conformation preventing flexibility / dissociation that may lead to aggregation. ii) DMPG also contributes to prevention of aggregation due to electrostatic repulsion between the negatively charged lipids on neighboring nanodiscs.

[1] T.H. Bayburt et al.: Nano Letters 2 (2002) 853.
[2] N. Skar-Gislinge and J.B. Simonsen et al.: JACS 132 (2010) 13713.

1193-Plat
The HSP70 Interaction with Phosphatidyl Serine on Membranes is the Initial Step its Release Into the Extracellular Medium
Jonathan Okerblom[1], Michael R. Williams[1], David M. Cauvi[2], Diana Schlamadinger[3], Judy Kim[3], Nelson Arispe[4], **Antonio De Maio**[2].
[1]IMSD Program, University of California, San Diego, La Jolla, CA, USA, [2]School of Medicine, University of California, San Diego, La Jolla, CA, USA, [3]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA, USA, [4]Department of Anatomy, Physiology and Genetics, Uniformed Services University, Bethesda, MD, USA.

Expression of heat shock proteins (hsp) is part of the universal cellular response to stress. The cytoprotective role of these proteins has been correlated with their chaperone function within the cytosol. In addition, hsp have been detected on the surface of stressed cells as well as in the extracellular medium. These extracellular hsp appear to play signaling role in the activation of the systemic response to stress. The question that arises is how hsp that do not display any consensus secretory signal or hydrophobic domains are inserted into membranes and secreted into the extracellular medium. We have previously shown that Hsp70, the major inducible hsp, was released embedded into the membrane of export or extracellular vesicles (ECV). The possible mechanism for Hsp70 insertion into membranes was investigated. We found that Hsp70 displayed a high specificity for phosphatidyl serine (PS) on the membrane, even if this lipid is combined with larger amounts of other phospholipids. The interaction of Hsp70 with PS was demonstrated by insertion into liposomes, changes in tryptophan fluorescence after exposure to artificial lipid membranes, and fluorophore leakage from liposomes. In addition, we showed extracellular Hsp70 bound to cells displaying PS on the surface, but not to surface PS negative cells. We propose that insertion of Hsp70 into membranes is a spontaneous process requiring the presence of PS. Therefore, we suggest that the insertion of Hsp70 within cellular membranes is the initial step in the export of this signaling molecule into the extracellular environment.